IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

TITLE: HIGH YIELD PROCESS FOR PRODUCING THEAFLAVINS AND PRODUCTS OF SUCH PROCESS

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CITATION TO PARENT APPLICATION(S)

This application is a continuation-in-part of co-pending U.S. Patent Application 10/625,500, which was a continuation-in-part of U.S. Application 09/721,438 (now U.S. Patent No. 6,602,527), from which priority is claimed pursuant to 35 U.S.C. 120.

BACKGROUND OF THE INVENTION

1. Field of The Invention

The present invention relates to the synthesis of highly beneficial theaflavins from substrates yielded from green tea leaves, and to the products of such process.

2. Background Information

Theaflavins (TFs) are colorless catechin dimers that significantly contribute to the brown-orange color and astringency of black tea liquors when oxidized. Generally, TFs may be formed through the polyphenol oxidase (PPO)-dependent oxidative polymerization of green tea polyphenols (GTP) or flavanoids during fermentation of green tea to black tea. TFs are primarily composed of a mixture of theaflavin, theaflavin-3'-O-gallate, theaflavin-3-3'-di-O-gallate and theaflavin-3-gallate.

TFs have highly significant anti-oxidant properties, as well as significant anti-inflammatory antimicrobial and

antiviral activity. Specifically, TFs are reported effective against various diseases, including cancer, cardiovascular and cerebrovascular diseases, diabetes, hypercholesterolemia, etc. as a cancer chemopreventive agent, as a photoprotective agent, as a protective agent against UVB-induced skin damage, as an inhibitor of cell transformation and PhIPO-induced mutagenicity.

The present invention provides a uniquely effective, high yield process for specifically producing theaflavins extracts from green tea polyphenols (GTP), a product which can then be incorporated into nutritional supplements and even topical preparations. In the latter case, such would be useful in the prevention of UV-related skin damage and ultimately of skin cancer.

The present method results from research, one product of which was the realization that any presumption of an equivalence in beneficial characteristics of green and black tea polyphenols (based on studies that find the benefits of the two teas to be roughly equivalent), and a resulting belief, among some, that black tea presents nothing more beneficial than green tea, is merely the result of greatly differing concentrations of beneficial compounds respectively green and black tea.

The theaflavins of black tea are demonstrably of greater value in the maintenance of health, but appear in conventionally produced black tea in substantially lower concentrations than the corresponding, and differently structured polyphenols in green tea (principally epigallocatechin gallate ("EGCG")). When equal quantities are compared, black tea theaflavins (produced through oxidation of green tea polyphenols) are considerably more beneficial than the green tea polyphenols. Yet, as alluded to above, conventionally produced black tea contains far lower levels of theaflavins than would be desirable, else the benefits of black tea theaflavins, compared to those of green tea polyphenols, would have been more clearly evident from prior studies.

Presently, black tea theaflavins are produced when tea leaves are rolled such that phenolase in the tea leaves (remaining separate from the green tea polyphenols in the unadulterated tea leaf) is forcibly mixed with the green tea polyphenols, by which the latter is oxidized, in part, to form black tea theaflavins. However, the present production of black tea favors over-oxidation to form highly condensed tannins, rather than the more desirable, beneficial theaflavins.

It naturally follows, that it would be highly desirable to provide some methodology by which optimally high yields of black tea theaflavins may be produced from green tea polyphenols ["GTP"]).

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a health-promoting, antioxidant composition produced through a novel and unobvious oxidative processing of green tea polyphenols.

It is an object of the present invention to provide a health-promoting composition produced through a novel and unobvious oxidative processing of green tea polyphenols.

It is an object of the present invention to provide a chemopreventive composition produced through a novel and unobvious oxidative processing of green tea polyphenols.

It is an object of the present invention to provide a photoprotective composition produced through a novel and unobvious oxidative processing of green tea polyphenols.

It is an object of the present invention to provide a composition which is inhibitive of PhIP0-induced mutagenicity and which is produced through a novel and unobvious oxidative processing of green tea polyphenols.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention is of the high yield production of beneficial, theaflavins, the natural counterpart of which are characteristic constituents of black tea, yet appearing in black tea (when conventionally produced) in far lower levels than would be desired. The practice of such invention involves the use of mushroom.

Throughout this discussion, the words "synthetic" or "synthesized" are used. It is to be understood that this should not be interpreted as indicating a chemical difference between theaflavins produced by conventional means, versus the presently disclosed method, but merely that which is produced is "synthesized" using the new method of the present invention.

Mushroom tyrosinase is a type of polyphenol oxidase and, most notably, will oxidize green tea polyphenols to produce theaflavins. Green tea leaves or the polyphenols contained in such are exposed to a mushroom tyrosinase enzyme that readily converts the catechins into theaflavins under typical fermentation conditions. The ideal parameters are characterized in that polyphenolic substances contained in moist fresh tea leaves contain a moisture content of at least 20% by weight based upon the dry weight of tea solids contained in the moist leaves. The temperature is maintained between about 20-50.degrees C at a pH between about 4.0 - 6.0. The reaction is executed by distributing a mushroom

tyrosinase or some form thereof, such as finely ground mushrooms, on the tea leaves in a fluidized bed or similar unit operation to achieve even distribution of the mushroom tyronsiane upon the active sites of the tea leaves or tea polyphenols. For dry green tea, a mushroom slurry can be used to soak the leaves. The oxidation duration is 10 to 360 minutes supplying oxygen through a number of means either naturally or in some mechanical form. The fermentation end result is a highly concentrated theaflavin slurry.

The fermentation step will not necessarily have converted all the tea polyphenols into theaflavins and the final concentration of such will be dependent on the time or duration of the reaction along with the temperature and pH conditions as well as the amount of mushroom tyrosinase employed. It is the intent of the present invention to achieve a high theaflavin concentration and allow other polyphenols or catechins to remain present within the final slurry so as to attain a number of different slurry profiles as feedstock into the extraction steps and ultimately into the final end product.

The slurry can be dried and stored for future use or filtered and send directly to solvent extraction where a typical hydrocarbon based solvent such as ethyl acetate is used to extract the theaflavins other solvents or alcohols can be employed if one may desire to remove the caffeine or other sensitive compounds for example. The extract is then dried via vacuum evaporation or other suitable drying techniques. Further extraction purification can also be employed by conventional chromatographic techniques with an

alcohol solvent to achieve a highly purified theaflavin / polyphenol extract. Other conventional techniques to those skilled in the art may also be employed to achieve a highly purified extract.

The method of the present invention uses mushroom tyrosinase in a manner which produces optimum yields of TFs through controlled oxidation of green tea polyphenols. While examples that follow utilize commercially available, extracted mushroom tyrosinase and green tea polyphenols, it should be understood that the present invention encompasses the use of such substances from any source. In fact, outside of the analytical proof-of-concept context in which precise quantity control and related product measurements are required, the mere, temporary mixing of mushrooms (source of mushroom tyrosinase) and green tea leaves (source of green tea polyphenols) in a suitable buffering solution will achieve the intended results. Such an approach is the most likely, commercially viable mode of practicing the present invention, in view of the relative economies of using extracts verus mixing of the natural sources for the reagents. Upon review of the present disclosure, suitable scale-up in a commercial context will be apparent to persons skilled in relevant arts.

EXAMPLES

The following examples are illustrative of the present invention and parts and percentages are by dry weight unless otherwise indicated. Theaflavins were quantified by HPLC, and used mushroom tyrosinase and standard theaflavins from SIGMA-ALDRICH CORPORATION. It should be noted that these examples are only

that -- examples. A wide range of conditions, such as time and units of enzyme, can be used because more time in process will offset a lesser enzyme presence, and vice versa.

EXAMPLE I

100 mg green tea polyphenols in 50 ml acetate buffer (100mM, pH6.5) was mixed with 25,000 units of tyrosinase and the mixture was kept at room temperature in dark for 30 minutes. The reaction mixture was extracted with 100ml ethyl acetate 4 times. The combined extracts were evaporated under reduced pressure with a rotary evaporator to remove ethyl acetate. The residue was dissolved in water and applied to HPLC separation. Waterman ODS semipreparative column was employed.

HPLC mobile phase contained solvent A (5% methanol in 100mM acetate buffer, pH4.8) and solvent B (70% methanol in 100mM acetate buffer, pH4.8). Gradient method at 25°C as follows: 0-5 min (100%A), 5-15 min (100%A to 75%A and 25%B), 15-40 min (75%A and 25%B to 50%A and 50%B), 40-50 min (50%A and 50%B to 30%A and 70%B), 50-55 min (30%A and 70%B to 100%A), 55-60 min (100%A) and 61 min stop of run. The theaflavins fraction was collected and identified by their retention time and co-chromatography with authentic TFs.

From 100mg of crude green tea polyphenols, 41mg of TFs was obtained.

EXAMPLE II

100 mg green tea polyphenols in 50ml acetate buffer (100mM, pH6.0) was mixed with 20,000 units of tyrosinase and the mixture was kept at room temperature in dark for 25 minutes. The reaction mixture was extracted with 100ml ethyl acetate 4 times. The combined extracts were evaporated under reduced pressure with a rotary evaporator to remove ethyl acetate. The residue was dissolved in water and applied to HPLC separation.

From 100mg of crude Green tea polyphenols, 35mg of TFs was obtained.

EXAMPLE III

200 mg green tea polyphenols in 70ml acetate buffer (100mM, pH6.8) was mixed with 30,000 units of tyrosinase and the mixture was kept at room temperature in dark for 35 minutes. The reaction mixture was extracted with 100ml ethyl acetate 4 times. The combined extracts were evaporated under reduced pressure with a rotary evaporator to remove ethyl acetate. The residue was dissolved in water and applied to HPLC separation.

From 200mg of crude green tea polyphenols, 92mg of TFs was obtained.

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The theaflavins produced according to the present invention may be incorporated into nutritional supplements and even topical creams, the latter for preventing UV-related skin damage through sun exposure.

In the case of nutritional supplements, the present synthesized products may be incorporated into capsules or

tablets, and administered orally. There is no specific dosage which is demonstrably better or worse than another. A capsule or tablet of within conventional size ranges, if consisting solely of oxidized extracts of the tea leaves here described, will provide a quite therapeutic dosage of theaflavins, and a much more potent such dosage than any extract of any tea leaves produced by any known method or process. Clearly, however, such extracts may well be combined with other nutritional supplement agents, in which case a perhaps proportionately less beneficial effect may be expected. One thing is certain, the theaflavins produced by the present process are health-promoting compounds, and the enhancement of their production provided by the instant process is beneficial in enabling the efficient delivery thereof.

The synthetically produced black tea theaflavins have proven to be as effective as theaflavins produced by conventional black tea processing. The present inventors have evaluated the theaflavins so produced as described hereafter.

TRIALS

To evaluate the preventative characteristics of the theaflavins produced according to the present method, the lower back of healthy adult human volunteers received a single topical application of theaflavins produced by the present process (0.2mg/cm^3) in 5% Tween 80), and 30 minutes later, were exposed to 120 mJ/cm^2 of UVB. 24 hours after UVB

exposure, sunburn severity indices were devised as area (cm^2) times intensity of red color lesion (0, no lesion; 1, barely detectable erythema; 2, moderate erythema; 3, bright erythema).

The following demonstrate the observed UVB damage preventative results:

TREATMENT	SUNBURN INCIDENCE (%)	SUNBURN SEVERITY
TF Alone	0	0
Vehicle + UVB	100	4.8 ± 1.1
Synth. TFs (0.2mg) + UVB	30	0.4 ± 0.4 (91%↓)
Conventional TFs	20	0.2 ± 0.5 (96%↓)

$P<0.01$

To evaluate the therapeutic characteristics of the theaflavins produced according to the present method, the lower back of healthy adult human volunteers received a single dosage (120 $mJ/cm^{2)}$ of UVB. Immediately, and 4 hours after UVB exposure, the exposed sites twice received topical application of either synthetized or conventionally-produced theaflavins (0.2mg/cm^3 in 5% Tween 80). 24 hours after UVB exposure, sunburn severity indices were evaluated as in the first trial.

The following demonstrate the observed UVB damage therapeutic results:

TREATMENT	SUNBURN INCIDENCE (%)	SUNBURN SEVERITY
TF Alone	0	0
Vehicle + UVB	100	4.8 ± 1.1
Synth. TFs (0.2mg) + UVB	80	1.2 ± 0.7 (75%↓)
Conventional TFs	50	1.4 ± 1.6 (71%↓)

$P<0.01$

The above results indicate that the synthesized theaflavins are effectively interchangeable with conventional theaflavins, with respect to efficacy. This interchangeability presumptively extends to other therapeutic contexts, such as in nutritional supplements.

In view of the foregoing, it is clear that practice of the present invention will yield a product, the use of which will yield a highly beneficial substance which can be used in both ingested and topical applications. This, in turn, represents a "painless" source of chemorepressive and sunburn resistance substances for consumption by consumers.

Although the invention has been described with reference to specific embodiments, this description is not meant to be construed in a limited sense. Various modifications of the disclosed embodiments, as well as alternative embodiments of the inventions will become apparent to persons skilled in the art upon the reference to the description of the invention. It is, therefore, contemplated that the appended claims will cover such modifications that fall within the scope of the invention.